American Journal of Pathology, Vol. 135, No. 5, November 1989

An Immunohistochemical Study of Neuropeptides and Neuronal Cytoskeletal Proteins in the Neuroepithelial Component of a Spontaneous Murine Ovarian Teratoma

Primitive Neuroepithelium Displays Immunoreactivity for Neuropeptides and Neuron-Associated β-Tubulin Isotype

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Approximately one third of the female mice of the LTXBO strain develop spontaneous ovarian teratomas. These tumors contain a large neuroepithelial component, which includes primitive neural structures resembling embryonic neural tubes (medulloepithelial rosettes), ependymoblastic and ependymal rosettes, neuroblasts, mature ganglionic neurons, myelinated neurites, and astrocytes. The purpose of this study was to characterize these tumors according to the immunohistochemical location of some well-characterized trophic and regulatory neuropeptides and neurotransmitters, several neuronal-associated cytoskeletal proteins, and other proteins indicative of neuronal and glial differentiation. Medulloepithelial rosettes showed focal serotonin-like, opioid peptide-like and γ-amino butyric acid-like immunoreactivity, and displayed immunostaining for the neuron-associated class III β-tubulin isotype. The mature ganglion cells were also immunoreactive for these markers, and, in addition, for somatostatin, cholecystokinin, bombesin, glucagon, vasoactive intestinal peptide, and neuropeptide Y. Mature ganglion cells were also immunoreactive for proteins associated with the neuronal cytoskeleton (including microtubule-associated proteins, MAP2 and tau, and higher molecular weight phosphorylated and nonphosphorylated neurofilament subunits), neuron-specific enolase, and synaptophysin. Undifferentiated stem cells, ependymoblastic and ependymal rosettes, and astroglia all stained with a monoclonal antibody that recognizes all mammalian β-tubulin isotypes, but did not react with antibodies to neuronal-associated cytoskeletal proteins or neuropeptides. Neuropeptide-like immunoreactivity and demonstration of the class III β-tubulin isotype indicate early neuronal commitment in neoplastic primitive neuroepithelium. These patterns of immunoreactivity closely follow those encountered in the normal neurocytogenesis of the mammalian and avian forebrain, and increase the precision with which the early stages of progressive neuroepithelial differentiation can be analyzed in human embryonal tumors of the CNS. (Am J Pathol 1989, 135:801–813)

In recent years, considerable attention has been given to the identification and distribution of neuropeptides, neurotransmitters, and neuron-associated cytoskeletal proteins in the developing and mature central nervous system

Supported by research grant CA 31271 from the National Cancer Institute, Graduate Neuropathology Training grant T32 NS 7236 from the National Institute of Neurological and Communicative Disorders and Stroke, US Department of Health and Human Services, and American Cancer Society grant CD 344.

Accepted for publication July 12, 1989.

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(CNS).[1-4] Our knowledge of the distribution and expression of those substances in neoplasms of the central and peripheral nervous systems, however, is still limited.[5-10] This is due in part to the relative rarity of CNS tumors composed of neuronal elements and in part to the scarcity of appropriately fixed and embedded tissues for the demonstration of neuropeptides and neurotransmitters.[11-13] An animal model of CNS tumor with divergent neuroepithelial differentiation may therefore be a useful system for overcoming those limitations, because it permits a systematic evaluation of immunohistochemical markers in elements with different degrees of maturation.

Spontaneous ovarian teratomas are very rare in rodents.[10,14,15] However, approximately 30% of the female mice of the LT strain and LTXBO substrain develop spontaneous ovarian teratomas by age 6 months (reference 14 and LC Stevens, personal communication). These tumors display a large component of neuroectodermal tissues, which include a variety of neuroblastic, glial, and ependymal elements, ranging in differentiation from primitive neuroepithelial structures to mature neurons.[14,15] The neoplasms are rich in areas of immature neuroepithelial tissue that morphologically resemble those found in the rare but important group of embryonic human CNS tumors, such as the medulloepithelioma, ependymoblastoma, medulloblastoma, and neuroblastoma. Recently, we studied eight spontaneous ovarian teratomas in the LTXBJ strain of mice and presented evidence that immunohistochemical reactivity for several neuron-associated cytoskeletal proteins can be demonstrated in the neoplastic neuroepithelial elements of the tumor[10]: of interest, the most primitive elements displayed immunoreactivity for the class III neuron-associated β-tubulin isotype. However, all the tumors in that study were routinely fixed in buffered formalin, thus preventing the immunohistochemical demonstration of other markers of neuroepithelial differentiation, such as neuropeptides and neurotransmitters or the cytoskeletal proteins, tau and vimentin, which require special processing conditions.

For the present study, we collected 18 ovarian teratomas arising in female mice of the LTXBO strain and, by using several fixative methods, demonstrated the immunohistochemical location of neuropeptides, neurotransmitters, and a larger panel of neuronal-associated cytoskeletal proteins. Our results showed that these neuroepithelial elements displayed distinct patterns of immunostaining that varied according to their degree of maturation. In particular, focal immunoreactivity of the primitive neuroepithelium for serotonin, γ-amino-butyric acid (GABA), opioid peptides, and for the class III β-tubulin isotype indicated its early commitment to the neuronal lineage, and increased the precision with which the early stages of progressive neuroepithelial differentiation could be defined in this embryonal tumor system.

Materials and Methods

Ten pairs of LTXBO mice were provided by Dr. Leroy Stevens, Jackson Laboratories, Bar Harbor, Maine. In our laboratory, these animals were maintained as an inbred colony for four generations during an 18-month period. Female mice were carefully observed at regular intervals, and animals were killed when signs of an abdominal tumor or ascites developed or when they were 6 months old. Each female mouse was autopsied and the ovaries promptly dissected and fixed, regardless of whether or not a macroscopic tumor was found. A total of 110 female mice were studied. Seventy animals did not develop tumors, and the ovaries did not show gross or microscopic abnormalities. Twenty-two female mice developed microscopic or very small ovarian tumors (11 in the right ovary, nine in the left, and two bilaterally) and were not included in this study. Eighteen larger tumors were analyzed in detail for the present report; they were mostly solid neoplasms, with an average measurement of approximately 2 cm in diameter.

In preliminary studies, several fixatives were tested in tissues from control mice to determine the best methods to preserve both histologic detail and antigenic reactivity. Of the fixatives tested [4% buffered aqueous formaldehyde, Bouin's solution, Zamboni's fixative,[16] 4% paraformaldehyde, and 70% ethanol], Bouin's solution consistently yielded superior results. Routine paraffin embedding did not alter antigenic preservation and yielded histologic detail that was superior to that obtained with cryostat sections.

Ovarian tumors were fixed for approximately 18 hours at room temperature in Bouin's solution and were subsequently embedded in paraffin. All normal mouse tissues used as controls were processed after similar procedures. Brain tissues (strain LTXBO, age 6 months) employed as controls were fixed by immersion in Bouin's solution or by perfusion through the aorta with chilled normal saline, followed by Zamboni's fixative for 30 minutes before dissection and postfixation overnight in Bouin's solution. Paraffin sections were stained with hematoxylin and eosin (H&E), phosphotungstic acid hematoxylin (PTAH), and Luxol fast blue for myelin sheaths.

Immunohistochemistry

Sections from all tumors and control tissues were immunostained according to the peroxidase-antiperoxidase method,[17] using a panel of monoclonal antibodies and rabbit polyvalent antisera (Table 1). All sections were deparaffinized in xylene for 10 minutes; after rehydration through graded ethanols and blockage of the endogenous peroxidase activity with hydrogen peroxide (0.5% in

Table 1. *Monoclonal Antibodies and Polyclonal Antisera Used for the Immunohistochemical Characterization of the Neuroepithelial Component of Spontaneous Ovarian Teratomas in the LTXBO Strain of Mice*

Antigen		Dilution	Source
Polyclonal antisera to neuropeptides and neurotransmitters			
γ-amino butyric acid (GABA)		1:500	Amac, Inc., Westbrook, ME
β-endorphin		1:200	Amersham Corp., Arlington Heights, IL
Met-enkephalin		1:250	Amersham
Somatostatin		1:500	Dako Corp., Santa Barbara, CA
Bombesin-gastrin-releasing peptide		1:250	Amersham
Vasoactive intestinal peptide (VIP)		1:250	Amersham
Cholecystokinin octapeptide-gastrin		1:250	Amersham
Neuropeptide Y		1:250	Amersham
Glucagon		1:500	Dako
Insulin		1:500	Dako
Serotonin MAb Clone 5HT-H209		1:50	Dako
Monoclonal antibodies to neuronal-associated cytoskeletal proteins			
Clone	Antigen		
TUJ1	Class III β-tubulin isotype (neuron-associated)	1:100	A. Frankfurter
AP18	Microtubule-associated protein 2	1:100	A. Frankfurter
Tau-1	Microtubule-associated protein Tau	1:100	A. Frankfurter
SMI 31	Phosphorylated high and middle molecular weight neurofilament subunits (NF-H/M)	1:1000	Sternberger-Meyer Immunocytochemicals Inc., Jarrettsville, MD
SMI 32	Nonphosphorylated, phosphorylation-dependent NF-H/M	1:1000	Sternberger-Meyer
SMI 33	Phosphorylation-independent, NF-H/M	1:1000	Sternberger-Meyer
Tp-NFP1A3	Phosphorylated, NF-H/M	1:100	V. P. Collins
Monoclonal antibodies to other cytoskeletal proteins			
Clone			
TU27	All mammalian β-tubulin isotypes	1:100	A. Frankfurter
V9	Vimentin	1:200	Dako
Antibodies to other neuronal or glial proteins			
Synaptophysin (MAb, Clone SY38)		1:100	Boehringer Mannheim BgmH, Mannheim, West Germany
Neuron-specific enolase		1:1000	P. J. Marangos, Gensia Pharmaceutical, San Diego, CA
Glial fibrillary acidic protein		1:1400	L. F. Eng, Stanford University, Stanford, CA
S-100 protein		1:2000	Dako
Antibodies to myelin-associated proteins			
Myelin-associated glycoprotein (MAb Clone B11 F7)		1:50	R. H. Quarles, National Institutes of Health, Bethesda MD
Myelin basic protein		1:1000	Dako

methanol for 30 minutes), three-step peroxidase-antiperoxidase reactions were carried out using polyvalent antisera and monoclonal antibodies. Before the application of the primary monoclonal antibodies or polyclonal antisera, specimens were saturated in 20% normal rabbit serum (Dako Corporation, Santa Barbara, CA) or 20% normal swine serum (Dako) for 30 minutes at room temperature, respectively.

Sections were then incubated at 4 C for 18 hours with the primary antibody or antiserum. After application of the polyclonal antisera, sections were exposed to swine anti-rabbit immunoglobulins (Dako) (diluted 1:50), and rabbit peroxidase anti-peroxidase complexes (Dako) (diluted 1:100) sequentially applied for 20 minutes at room temperature. After the application of the monoclonal antibodies, sections were incubated with rabbit anti-mouse immunoglobulins (Dako) (diluted 1:50) and mouse monoclonal peroxidase-antiperoxidase complexes (Dako) (diluted 1:100) sequentially applied at room temperature for 20 minutes. All reagents were diluted in 0.05 M Tris-buffered saline (TBS), pH 7.6, containing 1% normal swine serum. For the demonstration of neuropeptides and neurotransmitters, all antisera were diluted in TBS containing 0.1% Triton X (Sigma Chemical Co., Saint Louis, MO). The immunohistochemical reactions were developed either in freshly prepared 3,3′-diaminobenzidine tetrahydrochloride (DAB) (Sigma), 5.0 mg diluted in 6.5 ml of 0.05 M TBS containing 0.015% of hydrogen peroxide, or in 10 mg of 3-amino-9-ethylcarbazole diluted in 5 ml of 2.5 M acetate buffer, pH 5.0, containing 0.05% of hydrogen peroxide. In

addition, a double-labeling technique for the simultaneous demonstration of the neuron-associated β-tubulin isotype and glial fibrillary acidic protein was carried out using a double stain kit (Universal Double Stain Kit, Dako catalog number K665).

Sections were lightly counterstained with hematoxylin, except after immunostaining for some of the peptides and transmitters.

Control Procedures Employed for Immunohistochemistry

The specificity of the immunohistochemical stains for neuropeptides was tested using positive and negative controls and procedures of preabsorption of the primary antisera detailed elsewhere.[18,19] As positive controls, the following tissues were stained in parallel: mouse jejunum for serotonin and cholecystokinin (CCK); mouse pancreas for somatostatin, glucagon, and insulin; human pituitary for β-endorphin and met-enkephalin; mouse intestinal myenteric plexus for vasoactive intestinal peptide (VIP); adult mouse brain for γ-amino butyric acid (GABA, see reference 20 for characterization of this antiserum) and neuropeptide Y (NPY); and human small cell carcinoma of the lung for bombesin. All mouse tissues were obtained from 6-month-old LTXBO mice. Negative controls were obtained by replacing the first antibody with nonimmune mouse IgG1-kappa or IgG2a (for Mabs) (Sigma) or with nonimmune rabbit immunoglobulins (for polyclonal antisera) (Dako). These negative controls were used at the same concentration, temperature, and times of incubation as were the corresponding primary antibodies.

In addition, liquid phase absorption studies[18,19] were carried out and consisted of adding 10 to 100 nmol of the corresponding antigen to 1 ml of optimally diluted antiserum (Table 1), followed by incubation at 4 C for 24 hours. The antigens used were as follows: somatostatin; VIP, porcine sequence; CCK-8 (fragment 26-33, nonsulfated); glucagon, crystalline; neuropeptide Y, human sequence; bombesin; leucine and methionine-enkephalin (both for anti-met-enkephalin antiserum); β-endorphin (β-lipotropin fragment 61-76), human; serotonin hydrochloride; and GABA. All antigens were synthetic except glucagon, which was obtained from porcine pancreas, and all were provided by Sigma Chemical Co. Absorption of the primary antisera resulted in complete abolition of specific staining in tissue section controls, except for absorption of the met-enkephalin antiserum with leu-enkephalin; specific immunostaining was not affected, thus indicating that this antiserum did not cross-react with leu-enkephalin.

The specificity of the immunohistochemical stains for cytoskeletal, myelin-associated and other proteins indicative of glial or neuronal differentiation was tested using the following positive controls stained in parallel: adult mouse brain (strain LTXBO, age 6 months) for MAbs TUJ1, TU27, SMI 31, SMI 32, SMI 33, AP18, Tau-1, B11F7 (see reference 21 for characterization of this MAb), and SY38, and for polyclonal anti-neuron-specific enolase (NSE) and myelin basic protein (MBP) antisera; surgical specimens of well-differentiated human astrocytomas for glial fibrillary acidic (GFA) and S-100 proteins; and mouse adult kidney (strain LTXBO, age 6 months) for MAb V9 (anti-vimentin). Negative controls were identical to those used for immunostaining of neuropeptides.

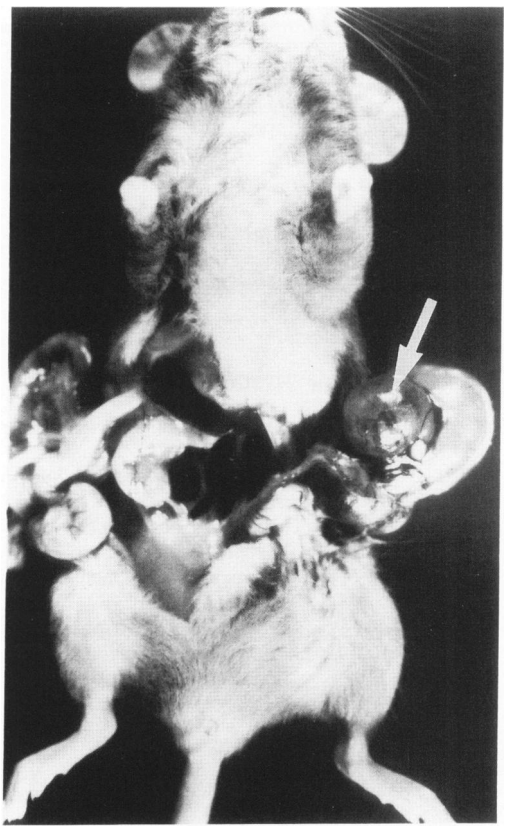

Figure 1. *A 3-month-old LTXBO female mouse developed a large, partially cystic tumor in the left ovary (arrow).*

Results

The 18 tumors analyzed in detail in this study contained a large and predominant neuroepithelial component and were mostly solid, bulky neoplasms composed of soft gray tissue (Figure 1). Nine developed in the right ovary, five in the left, and four were bilateral: their average diameter was 2.3 cm. Histologically they displayed all stages of neuroepithelial differentiation.

The histologic components found in these complex tumors were divided into six main stages in relation to their progressive neuroepithelial differentiation. These were 1)

Table 2. *Summary of Immunohistochemical Findings in the Neuroepithelial Component of Spontaneous Ovarian Teratomas in the LTXBO Strain of Mice*

	Stem cells	Medulloepithelial rosettes	Ependymoblastic and ependymal rosettes	Neuroblasts	Neurons	Astroglia
MAb TU27*	+	+	+	+	+	+
MAb TUJ1*	−	+	−	+	+	−
Vimentin	−	+	+	−	−	−
MAP2	−	−	−	+	+	−
Tau	−	−	−	+	+	−
NF epitopes*	−	−	−	−	+	−
NSE	−	−	−	−	+	−
Synaptophysin	−	−	−	−	+	−
GFAP	−	−	−	−	−	+
S-100	−	−	−	−	−	+
MBP, MAG	−	−	−	−	+	−
Serotonin	−	+	−	+	+	−
GABA	−	+	−	+	+	−
Met-enkephalin	−	+	−	+	+	−
β-endorphin	−	+	−	+	+	−
Somatostatin	−	−	−	−	+	−
Bombesin	−	−	−	−	+	−
Cholecystokinin	−	−	−	−	+	−
Neuropeptide Y	−	−	−	−	+	−
VIP	−	−	−	−	+	−
Glucagon	−	−	−	−	+	−
Insulin	−	−	−	−	−	−

* MAb TU27 recognizes an epitope present in all mammalian β-tubulin isotypes: Mab TUJ1 recognizes an epitope in the class III β-tubulin isotype (neuron-associated). NF epitopes were detected with MAbs SMI 31, SMI 32, SMI 33, and Tp-NFP1A3.

MAb, monoclonal antibody; MAP2, microtubule-associated protein 2; NF, neurofilament; NSE, neuron-specific enolase; GFAP, glial fibrillary acidic protein; MBP, myelin basic protein; MAG, myelin-associated glycoprotein; GABA, γ-amino butyric acid; VIP, vasoactive intestinal peptide.

undifferentiated stem cells; 2) primitive neuroepithelium, including medulloepithelial rosettes; 3) ependymoblastic rosettes; 4) ependymal rosettes; 5) neuroblasts; and 6) mature neurons and astroglia.

Observations are presented for each of these components and are summarized in Table 2.

Stem Cells

In all tumors, except those composed entirely of mature elements, there were areas consisting of sheets of undifferentiated stem cells with basophilic cytoplasm, vesicular nuclei with prominent nucleoli, and many mitotic figures. Immunohistochemically, stem cells reacted only with the MAb TU27 (Figure 2A, upper), which recognizes all mammalian β-tubulin isotypes and therefore immunostains different types of tubulin-rich structures. The tumors also contained small tubular and glandular structures whose ciliated epithelium also reacted with MAb TU27 (Figure 2A, lower). Stem cells and ciliated epithelium did not stain with MAb TUJ1 (Figure 2B), which recognizes the neuron-associated class III β-tubulin isotype, or with any of the other monoclonal antibodies or antisera to neuronal cytoskeletal proteins, neuropeptides, or neurotransmitters.

Medulloepithelial Rosettes

These structures were interpreted as a component of the most primitive elements displaying neuroepithelial differentiation. They consisted of circular or elongated tubular structures lined by a pseudostratified cylindrical epithelium, and possessed well-defined internal and external limiting membranes. Near the lumen, mitotic figures were abundant (Figure 2G, I). The overall appearance of the formations resembled an embryonic neural tube or the primitive structures found in the medulloepithelioma,[22] a rare but well-studied embryonal CNS tumor with divergent differentiation potential.

Some of the cells forming these rosettes were immunoreactive for serotonin (Figure 2C, D), met-enkephalin (Figure 2E), GABA (Figure 2F), and β-endorphin. For these substances, the pattern of immunoreactivity was delicate and could only be discerned in sections without counterstaining.

Cells of the medulloepithelial rosettes reacted focally for vimentin (Figure 2G, H). The rosettes were also positive with both anti-β-tubulin MAbs, but showed different patterns for each of them. With TU27, reactivity was diffuse and widespread (Figure 2I). With TUJ1, immunopositive cells were concentrated at the base of the primitive neuroepithelium near the external limiting membrane

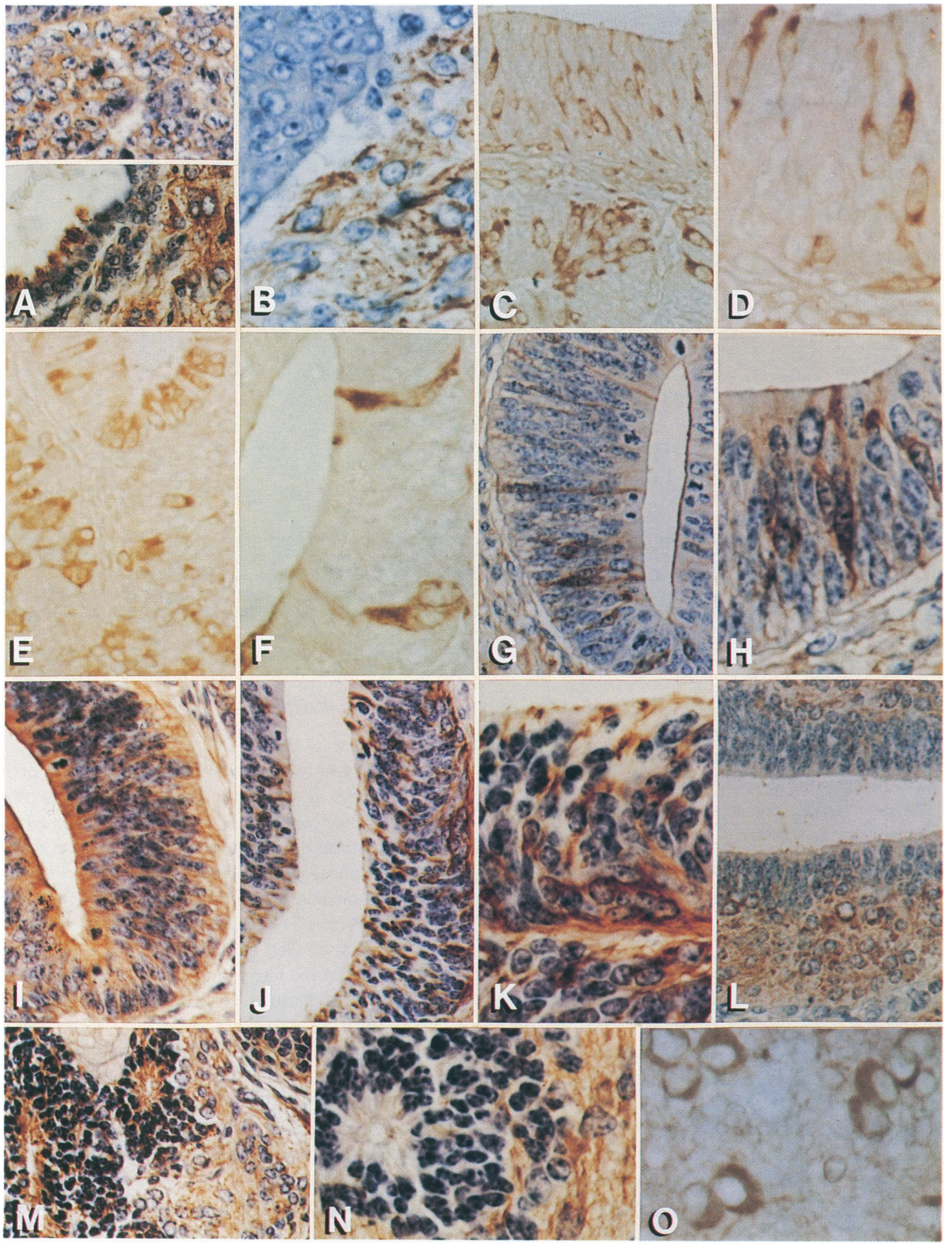
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Figure 2. *Immunohistochemical patterns of primitive stem cells and medulloepithelial and ependymoblastic rosettes.* **A:** *Stem cells were immunoreactive with MAb TU27 (upper field). This MAb also reacted with ciliated epithelium of teratomatous tubules and neuroblasts (lower field) (immunoperoxidase stain counterstained with hematoxylin; original magnification ×400).* **B:** *Stem cells (upper left) did not react for the class III associated β-tubulin isotype recognized by MAb TUJ1. In contrast, neuroblasts were strongly reactive (lower right) (immunoperoxidase stain counterstained with hematoxylin; original magnification ×400).* **C:** *Some cells of a medulloepithelial rosette displayed serotonin-like immunoreactivity. The lumen of the structure is at the top. Adjacent small neuroblasts were also immunopositive (lower half) (immunoperoxidase stain, no counterstain; original magnification ×200).* **D:** *A detail of Figure 2C shows delicate positivity for serotonin in linearly arrayed polar processes of cells of a medulloepithelial rosette. The lumen of the structure is at the top (immunoperoxidase stain, no counterstain; original magnification ×400).* **E:** *Cells of a medulloepithelial rosette (top) and adjacent neuroblasts (bottom) were immunoreactive for met-enkephalin (immunoperoxidase stain, no counterstain; original magnification ×200).* **F:** *Immunopositivity for γ-amino-butyric acid was focally present in cells of a medulloepithelial rosette (immunoperoxidase stain, no counterstain; original magnification ×400).* **G:** *Focal immunoreactivity for vimentin in a medulloepithelial rosette. Note the resemblance of the overall structure to the early stages of the embryonic neural tube. Mitotic figures in a juxtaluminar position can be seen (immunoperoxidase stain counterstained with hematoxylin; original magnification ×200).* **H:** *Detail of the previous figure showing vimentin positivity in cells of a medulloepithelial rosette (immunoperoxidase stain counterstained with hematoxylin; original magnification ×400).* **I:** *The neuroepithelium of a medulloepithelial rosette demonstrated diffuse positivity with MAb TU27 (immunoperoxidase stain counterstained with hematoxylin; original magnification ×400).* **J:** *With MAb TUJ1 immunoreactivity in medulloepithelial rosettes was focal and was more evident in cells at the base of the epithelium (right field) (immunoperoxidase stain counterstained with hematoxylin; original magnification ×200).* **K:** *Detail of the previous figure showing immunoreactivity with TUJ1 in numerous cells at the base of the epithelium of a medulloepithelial rosette. The lumen of the rosette is at the top (immunoperoxidase stain counterstained with hematoxylin; original magnification ×400).* **L:** *The epithelium of a medulloepithelial rosette did not immunostain for microtubule-associated protein 2. In contrast, note the positivity in adjacent small neuroblasts (immunoperoxidase stain counterstained with hematoxylin; original magnification ×200).* **M:** *Two ependymoblastic rosettes (left half) and adjacent neuroblasts (right half) were immunoreactive with MAb TU27 (immunoperoxidase stain counterstained with hematoxylin; original magnification ×400).* **N:** *An ependymoblastic rosette (left) did not react with MAb TUJ1. Note the immunopositivity in adjacent neuroblasts (right) (immunoperoxidase stain counterstained with hematoxylin; original magnification (×200).* **O:** *Small neuroblasts showing serotonin-like immunoreactivity in somata and short neurites (immunoperoxidase stain, no counterstain; original magnification ×400).*

(Figure 2J, K). The medullary epithelium did not stain with an anti-MAP2 MAb. However, neuroblasts surrounding the rosettes were positive (Figure 2L). MAbs to neurofilament (NF) epitopes or other neuronal or glial markers did not react with medulloepithelial rosettes.

Ependymoblastic Rosettes

Ependymoblastic rosettes consisted of primitive areas with small central lumina surrounded by stratified small cells with dense chromatin, abundant mitotic figures, and apical ciliated borders. These structures were identical to the rosettes found in the ependymoblastoma, a rare embryonal CNS tumor, and were interpreted as representing early ependymal differentiation.[23] In the tumors derived from the OTT-6050 mouse teratoma line, electron microscopy showed transitional features between the less-differentiated medulloepithelial rosettes and ependymoblastic rosettes.[24]

Cells in the ependymoblastic rosettes reacted with TU27 (Figure 2M) and focally for vimentin, but not with TUJ1 (Figure 2N), NSE (Figure 3O), or any of the other antibodies to neuronal and glial proteins or neuropeptides.

Areas of Ependymal Rosettes

These structures, similar to the rosettes found in human ependymomas, possessed wide circular or elongated lumina and were lined by a single or double layer of cuboidal cells in which small apical inclusions (blepharoplasts) could be seen with the PTAH stain. Ependymal rosettes were often found adjacent to immature ependymoblastic rosettes and, like the more primitive structures, immunoreacted with TU27, but not with TUJ1 or with other antibodies to neuronal cytoskeletal proteins and neuropeptides.

Neuroblasts

All tumors contained densely cellular areas composed of small or larger neuroblasts, identified by their round nuclei with a delicate chromatin pattern, small nucleoli, and delicate eosinophilic polar processes (neurites) forming a finely fibrillated matrix. Sheets of neuroblasts usually surrounded medulloepithelial rosettes and blended further afield with less densely cellular areas composed of large neurons.

Groups of neuroblasts reacted for serotonin (Figure 2O), GABA (Figure 3A), met-enkephalin (Figure 3B), and β-endorphin. They were also immunopositive with both TU27 and TUJ1 MAbs (especially in the neurites, Figure 2B) and for MAP2 (in the somata and neurites, Figure 3C). Focally, neuroblasts were immunoreactive for tau (Figure 3D), but did not react with MAbs to NF, synaptophysin or vimentin, or for NSE.

Mature Neurons and Astroglia

In all tumors, the largest component invariably consisted of areas of large mature neurons with vesicular nuclei,

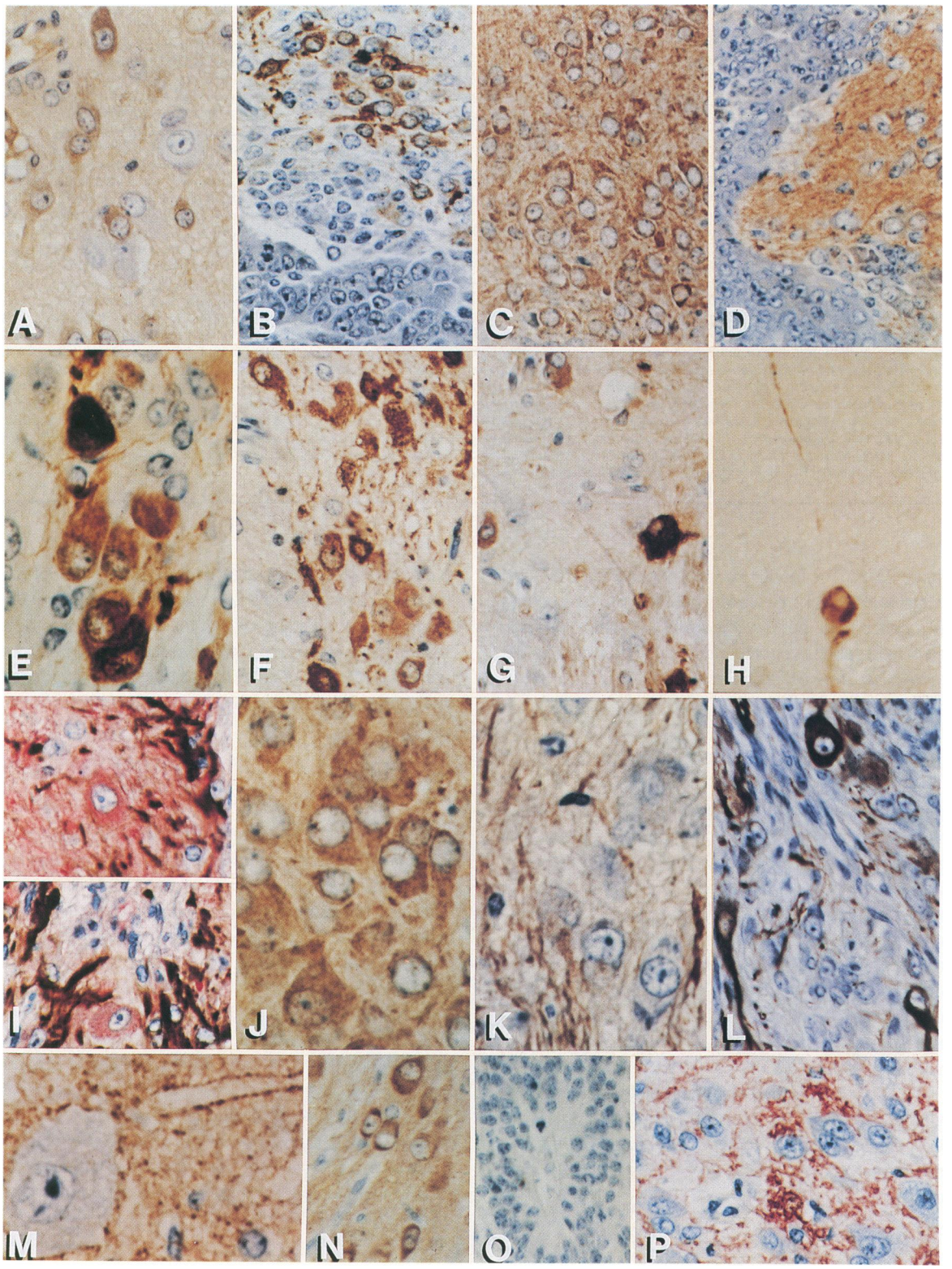
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Figure 3. *Immunobistochemical pattern of neuroblasts, neurons, and astrocytes.* **A:** *Some of the large neuroblasts showed immunoreactivity for γ-amino butyric acid (immunoperoxidase stain counterstained with hematoxylin; original magnification ×400).* **B:** *Neuroblasts displayed immunoreactivity for met-enkephalin. Note the negative immunoreactivity in adjacent stem cells (bottom) (immunoperoxidase stain counterstained with hematoxylin; original magnification ×200).* **C:** *Numerous neuroblasts were immunoreactive for microtubule-associated protein 2 (MAP2) (immunoperoxidase stain counterstained with hematoxylin; original magnification ×400).* **D:** *An area of neuroblasts displayed immunoreactivity for tau in neurites. Note the negative stem cells (left half) (immunoperoxidase stain counterstained with hematoxylin; original magnification ×400).* **E:** *A group of large neurons showed cytoplasmic immunoreactivity for somatostatin (immunoperoxidase stain counterstained with hematoxylin; original magnification ×400).* **F:** *Several large neurons displayed immunoreactivity for cholecystokinin in perikarya and neurites (immunoperoxidase stain counterstained with hematoxylin; original magnification ×400).* **G:** *Large neurons demonstrated bombesin-like immunoreactivity in perikarya and neurites (immunoperoxidase stain counterstained with hematoxylin; original magnification ×400).* **H:** *A solitary neuron showing immunoreactivity for neuropeptide Y in its perikaryon and in a long neurite (immunoperoxidase stain, no counterstain; original magnification ×400).* **I:** *In this double-labeling immunostain, which is a composite of two adjacent fields in the same preparation, large neurons showed positivity with MAb TUJ1 (red), whereas nearby astrocytes were immunoreactive for glial fibrillary acidic protein (brown) (Double-labeling immunostain with peroxidase [developed with diaminobenzidine] and alkaline phosphatase [developed with Fast Red TR] counterstained with hematoxylin; original magnification ×400).* **J:** *A group of large neurons displayed perikaryal immunostaining for MAP2 (immunoperoxidase stain counterstained with hematoxylin; original magnification ×400).* **K:** *Immunoreactivity with MAb SMI 31 displayed in neurites of large ganglion cells (immunoperoxidase stain counterstained with hematoxylin; original magnification ×400).* **L:** *With MAb SMI 32 large neurons showed immunoreactivity in their perikarya and processes (immunoperoxidase stain counterstained with hematoxylin; original magnification ×400).* **M:** *A large neuron displayed immunoreactivity for synaptophysin along the cell membrane covering the perikaryon and a thick neurite (immunoperoxidase stain counterstained with hematoxylin; original magnification ×400).* **N:** *Neurons showed immunoreactivity for neuron-specific enolase (NSE) (immunoperoxidase stain counterstained with hematoxylin; original magnification ×200).* **O:** *An ependymoblastic rosette near the neurons in Figure 3N was negative for NSE (immunoperoxidase stain counterstained with hematoxylin; original magnification ×200).* **P:** *Immunoreactivity can be seen for myelin basic protein in cell processes surrounding large neurons (immunoperoxidase stain developed with amino-ethyl carbazole counterstained with hematoxylin; original magnification ×200).*

prominent nucleoli, and perikaryal Nissl granules. These cells were dispersed in an abundant, delicately eosinophilic neuropile containing smaller cells with the morphologic characteristics of glia.

Singly arranged or small groups of neurons were immunoreactive for all the neuropeptides and neurotransmitters studied, with the exception of insulin. In general, perikarya and neurites were immunolabeled, with the exception of glucagon, in which case only neurites were immunopositive. The frequency of neurons labeled varied for each neuropeptide. Somatostatin- (Figure 3E), met-enkephalin-, serotonin-, and GABA-immunoreactive neurons were more often seen than those showing CCK (Figure 3F), bombesin (Figure 3G), NPY (Figure 3H), or VIP immunoreactivity.

Neuronal perikarya were strongly reactive with TUJ1 (Figure 3I) and TU27 and for MAP2 (Figure 3J), whereas tau was chiefly present in neurites. In addition, positivity for NF epitopes was detected in processes in the neuropile (especially with SMI 31 [Figure 3K] and Tp-NFP1A3) and in neuronal somata (especially with SMI 32, Figure 3L). The focal pattern of immunoreactivity for neuropeptides contrasted with the widespread positivity for neuron-associated cytoskeletal proteins.

Synaptophysin was detected as delicate granular deposits in the neuropile, and was especially evident on the cytoplasmic membranes of mature neurons (Figure 3M). Positivity for NSE consisted of reactive neuronal perikarya (Figure 3N) and neurites. Bundles of myelinated fibers were stained with Luxol fast blue and were immunoreactive for MBP (Figure 3P) and, more focally, for MAG. No reactivity for myelin proteins was detected in cell bodies.

Astrocytes, identified as such with hematoxylin and eosin (H&E) and PTAH stains, were also abundant in the areas of advanced neuronal maturation. They reacted strongly for GFA (Figure 3I) and S-100 proteins and with MAb TU27, but did not react with MAb TUJ1 or MAbs to neuronal-associated cytoskeletal proteins and vimentin, or for NSE. Reactivity for neuropeptides or neurotransmitters was not detected in glial cells. Using a double-labeling technique, reactivity with TUJ1 was restricted to neurons and neuroblasts, whereas GFA protein was detected only in cells with astrocytic morphology (Figure 3I). Cells coexpressing both markers were not found.

Discussion

Spontaneous germ cell tumors are very rare in laboratory mice. However, 1% of male mice of the 129 strain develop testicular teratomas[25,26] and 30% of female mice of the LT strain (of which the LTXBO strain is a substrain) develop ovarian teratomas by the age of 6 months.[14,26] Most of these rodent tumors, unlike those in humans, are benign and do not metastasize. In the LT strain, oocytes in the ovary undergo spontaneous parthenogenetic activation resulting in embryos that develop into teratomas.[14] These tumors contain a population of stem cells (also called embryonal carcinoma or EC cells), which are believed to be pluripotent and to differentiate into all the other cell types.[26,27] Teratomas in the LT strain and its substrains are remarkable for their large component of neuroepithelial elements.[14,15] We recently showed in tumors of the LTXBJ strain that these elements display different pat-

terns of immunohistochemical reactivity for neuronal-associated cytoskeletal proteins that vary according to their degree of maturation.[10] In this more comprehensive study in the LTXBO strain, we demonstrated that a pattern of immunohistochemical reactivity that parallels that observed for neuronal cytoskeletal proteins also exists for neuropeptides and neurotransmitters.

More than 200 different peptides, transmitters, and related substances were identified as proven or putative neuromediators in the mammalian CNS, and the list is continuously growing.[1,28] Consequently, our study was limited to a group of well-characterized peptides and transmitters whose distribution and expression during CNS ontogeny is relatively well documented. Although several reports identified these substances in neuroendocrine tumors,[29] there have been very few immunohistochemical studies of neuroepithelial tumors. This is due to the relative rarity of human or animal tumors with a predominant component of neuronal or neuroblastic elements, and to the stringent requirements for fixation and processing necessary to demonstrate these substances immunohistochemically.[12,13]

Most studies involving the distribution of neuropeptides in the mammalian CNS dealt with the rat cerebral cortex, particularly with the visual cortical area.[28,30] Whereas there is adequate information for some substances (monoamines), our knowledge concerning the development of neuropeptides and other neurotransmitters is still incomplete.[28,30,31] Although it is apparent that several of those substances are demonstrable before synaptogenesis and neurotransmission occur, it is still difficult to establish the precise time during development when they first appear. For example, in fertilized eggs, serotonin, norepinephrine, dopamine, and acetylcholine can be detected both biochemically and histochemically.[31] Nevertheless, it is well established that two of the earliest systems that appear in the embryonic mammalian CNS are those of GABAergic and serotonergic fibers. Both serotonin-[32] and GABA-immunoreactive[33] cells are found in the ventricular layer of the neural tube of the rat as early as embryonic day 13, ie, just after closure of the tube. Opiate systems also appear early; endorphins have been detected biochemically as early as embryonic day 13 in the rat, and enkephalin immunoreactivity can be detected in proliferating neuroepithelial cells, eg, in the external granular cell layer of the cerebellum, a day later.[34,35] In the present study, immunoreactivity for GABA, serotonin, and opioid peptides was detected in the medulloepithelial rosettes, thus indicating that these substances may be markers of early neuroblastic differentiation in primitive neoplastic neuroepithelium.

In contrast, we observed somatostatin, glucagon, NPY, bombesin, and CCK immunoreactivity in small groups of neuroblasts and neurons, but not in medulloepithelial rosettes. These peptides appear later during CNS development than GABA-, serotonin-, and opioid-immunoreactive systems.[28,30] In combined autoradiographic and immunocytochemical studies of the rat brain, somatostatin neurons were shown to originate through the period of cortical formation, migrate to their final destination, then mature morphologically and begin expressing somatostatin around the time of birth.[36] Neuropeptide Y-, VIP-, and CCK-immunoreactive systems also appear in the more advanced stages of CNS development.[28,30,37-39] Therefore, the neuroepithelial tissues of the LTXBO tumors express immunoreactivity for neuropeptides in a sequence that, to a large extent, recapitulates the pattern seen during CNS development.

Cells in the medulloepithelial rosettes of these tumors were focally positive for vimentin. In early stages of the embryonic neural tube, which the medulloepithelial rosettes resemble morphologically, vimentin appears to be the earliest intermediate filament to be expressed.[40,41] We also found focal positivity for vimentin in ependymoblastic rosettes; similar observations were reported in the ependymoblastic rosettes of human ependymoblastomas.[42]

The immunohistochemical detection of the neuronal-associated class III β-tubulin isotype in the medulloepithelial rosettes represents a significant finding. In recent years, important advances were made in our knowledge of the structure and expression of the tubulin isotypes (see reference 43 for review). Six functional mammalian β-tubulin genes were identified and sequenced.[43] The class III isotype is expressed in neurons but not in glia or non-neural cell types,[43] except for Sertoli cells of the testes.[10,44-46] In this study we employed TUJ1, a MAb that specifically recognizes the class III isotype, and compared the results obtained with those seen with TU27, a MAb that recognizes an epitope common to all mammalian β-tubulin isotypes and consequently reacts with β-tubulin in all cell types[10,44]; many different structures and tissues, such as stem cells, medulloepithelial and ependymoblastic rosettes, neuroblasts, neurons, glia, and ciliated epithelia reacted with MAb TU27. In contrast, reactivity with TUJ1 was restricted to cell bodies and processes of neuroblasts and neurons, and to some of the cells of the medulloepithelial rosettes, presumably indicating early neuroblastic differentiation. These results confirmed that the class III isotype is expressed predominantly in cells of neuronal lineage.[43,47]

Supporting the hypothesis that some of the cells forming the medulloepithelial rosettes may have an early commitment to a neuronal lineage, recent observations in the chick embryo showed that the class III β-tubulin isotype (detected immunohistochemically with the TUJ1 MAb) is present in placodal precursors of the trigeminal ganglion when these cells are still in the cuboidal epithelial stage.[48] We also localized this tubulin isotype in medulloepithelial

rosettes in the experimental tumors produced by the OTT-6050 line of mouse teratoma.[49]

Microtubule-associated protein 2 (MAP2) was detected in areas of small neuroblasts and mature neurons in the LTXBO tumors. This neuron-associated MAP is transiently expressed in axonal neurites during early neuronal cytogenesis, but is only found in perikarya and dendrites in mature neurons.[50–55] In tumors, MAP2 was demonstrated in human neuroblastomas[5] and medulloblastomas,[56] and in *in vitro* cultures of retinoblastoma cells.[9,57] Immunoreactivity for tau, a microtubule-associated protein found predominantly in axons,[58] was also detected in neurites of neuroblasts and neurons in the LTXBO tumors.

In this study, we employed four MAbs to different epitopes of the NFP: SMI 31, which recognizes a phosphorylated epitope in the high (NF-H) and middle (NF-M) molecular weight neurofilament subunits[59]; Tp-NFP1A3, which reacts with a putative phosphorylated epitope of NF-H (reference 60 and Collins, unpublished data); SMI 32, which recognizes a nonphosphorylated/phosphorylation-dependent epitope in NF-H/M; and SMI 33, which is presumably a phosphorylation-independent epitope in the constant (homogenous) region of NF-H/M. MAbs SMI 31 and Tp-NFP1A3 both immunostain mainly axonal processes, whereas SMI 32 reacts mostly with cell bodies and dendrites.[59] In contrast to results for the class III β-tubulin isotype and MAP2, we found immunoreactivity with anti-NF MAbs only in areas of mature neurons. The absence of NF reactivity in the more immature elements correlates well with the finding that microtubules are detected in embryonic neurites before neurofilaments appear.[40,61]

Therefore, the sequence of expression of neuronal-associated cytoskeletal proteins in this tumor system appears to have the following pattern: medulloepithelial rosettes express the neuron-associated class III β-tubulin and vimentin; small neuroblasts are positive for the class III β-tubulin isotype, MAP2 and tau; and large neurons display reactivity for these neuronal microtubular proteins, as well as for NF epitopes.

In the mature areas of the tumors, two additional markers of neuronal differentiation were detected: synaptophysin and NSE. The former, a glycoprotein that is an integral component of synaptic vesicles, has become a well-established marker of neuroendocrine and neuronal differentiation in neoplasms.[62,63] The presence of synapses in these spontaneous murine ovarian teratomas was demonstrated ultrastructurally in the closely related LTXBJ and LTXLXBJ strains.[15] Neuron-specific enolase becomes detectable during normal development after neuronal migration is completed and synaptogenesis is initiated.[64] The anti-NSE antiserum that we used is directed against the γ subunit of enolase and was shown to react with non-neoplastic neurons and neuroendocrine cells but not with other cell types.[64]

Myelin basic protein and myelin-associated glycoprotein were also detected in the mature areas of the LTXBO tumors. The presence of myelin sheaths is unusual in human and experimental teratomas with neuroepithelial differentiation,[65] but has been documented by electron microscopy in the tumors of the related LTXBJ and LTXLXBJ mouse strains.[15]

In summary, the patterns of immunoreactivity displayed in the different neuroepithelial components of neoplastic neuroepithelium in this tumor closely follow those encountered in the normal neurocytogenesis of the mammalian and avian forebrain. The demonstration of immunoreactivity for serotonin, GABA, and opioid peptides, and for the class III β-tubulin isotype, which is indicative of the early neuronal commitment of these cells, increases the precision with which the early stages of progressive neuroepithelial differentiation can be analyzed in this tumor system.

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Acknowledgment

The technical assistance of Christopher Griggs, Beverly Livesay, and Ursula Miller is gratefully acknowledged. Sue E. Pearce provided expert secretarial assistance.